BIOCHEMICAL CONVERSION PROGRAM
ANNUAL REVIEW MEETING

at the

SOLAR ENERGY RESEARCH INSTITUTE

October 13-15, 1987

Overview of Enzymatic Hydrolysis Research

John D. Wright
Solar Energy Research Institute
1617 Cole Blvd.
Golden, CO 80401

Abstract

A successful process for converting lignocellulose to liquid fuels must make use of all three of the major components of the feedstock (cellulose, xylan from the hemicellulose, and lignin), plus most of the minor constituents as well (organic acids and extractives). Because cellulose is generally the largest fraction and because the processes used for the hydrolysis of cellulose affects all the other utilization processes, research on hydrolysis processes are the central component of the Biochemical Conversion/Alcohol Fuels Research Program.

Of all the parameters which may be used to describe a process, yield and product concentration are the most important. Therefore, because enzymatic hydrolysis with fungal enzymes holds the promise for the high yields and ethanol concentrations, as well as low capital and operating costs, enzymatic hydrolysis is our chief focus. All enzymatic processes for the utilization of cellulose have four major components: pretreatment, hydrolysis, fermentation, and enzyme production. The research carried out in each of these areas, as well as its rationale, is described in this section.

Alternate Enzymatic Hydrolysis Processes: Fungal and Bacterial

Two distinctly different types of enzymatic hydrolysis processes exist; those using enzymes produced by fungi, and those which use bacterial cellulases. Bacterial systems have received academic study in the U.S. at the Massachusetts Institute of Technology (Cooney et al 1987, Wang et al. 1986), Dartmouth (Lynd, Wolkin, and Grethlein 1986), and the University of Wisconsin among others, and the Institut Pasteur in France. Additionally, in Japan, the New Energy Development Corporation (NEDO), along with a consortium of private corporations and university researchers has targeted a bacterial process for extensive development with commercialization scheduled for 1992 (New Energy Development Organization 1987). In general, these projects use thermophilic bacterial, usually of the genus Clostridium, to carry out the hydrolysis of cellulose to sugars. These organisms have the further advantage that they can also simultaneously ferment the sugars to ethanol. Further, some thermophillic bacteria can also ferment xylose to ethanol. The disadvantages of these organisms are: they only produce ethanol concentrations of 1.5%, and because they have multiple metabolic pathways, they often produce several products other than ethanol, thus reducing the yield of the desired product. The attributes of these organisms for producing ethanol are described in an excellent review by Lynd (1987)

Fungal systems have received considerably more development. Fungal cellulases based on the organism Trichoderma reesei are sold in industrial quantities by several companies: Genenecor (U.S.), Novo (Denmark), Finnsugar (Finland), and Yakult-Honsha (Japan). Large pilot scale experiments aimed at commercialization are being carried out in France (ASCAF 1986). Yields of ethanol from cellulose in such processes are greater than 90%, and ethanol concentrations of 5% have been achieved. Fermentation of the xylose can be carried out with other organisms (even with a Clostridium bacteria if desired). Because of the higher yields, ethanol concentrations, and greater stage of

development, the Biochemical Conversion/Alcohol Fuels Program has focused its efforts on these types of processes.

Enzymatic Hydrolysis

All enzymatic hydrolysis processes consist of four major steps that may be combined in a variety of ways: pretreatment, enzyme production, hydrolysis, and fermentation. A schematic of the general process layout is shown in Figure 1.

Pretreatment

The key to increasing the digestibility of lignocellulose lies in increasing the cellulose surface area that is accessible to the enzymes. The internal surface area of wood is large, but only a small fraction is accessible to large molecules such as cellulase. Through prehydrolysis the hemicellulose fraction can be removed, thus enlarging the pore size and opening the structure to attack by the enzymes (Grethlein et al. 1984). Further, the degree of digestibility is almost directly proportional to the fraction of the xylan removed (Grohmann et al. 1985). Thus, all the major pretreatment options, dilute acid, steam explosion (Brownell and Saddler 1984), and organosolv processes (Holtzapple and Humphrey 1984), are acid-catalyzed removal of hemicellulose. Pretreatment research includes dilute acid pretreatment, organosolv pretreatment, and efforts to understand the interaction between pretreatment and hydrolysis. Steam explosion is not studied because it degrades a substantial amount of the hemicellulose into furan compounds.

Dilute acid pretreatment is being investigated because it is simple, effectively increases the digestibility of the biomass, and converts approximately 80% of the xylan into xylose for fermentation. Since the realization that enzymatic digestibility is to a large extent controlled by xylan removal, progress on this technology has been rapid, as the accumulated knowledge of acid hydrolysis technology can be applied. While this process is already well developed, perhaps the greatest improvement which remains is to increase the yield of xylose from xylan from the current value of 80%, without greatly increasing water or energy consumption. In the past year, research on this process at SERI has shown that the material produced in the pretreatment process must be smaller than one sixteenth of an inch in order for enzymatic hydrolysis to proceed at a rate which is not limited by diffusion of the enzyme into the interior of the particle. However, particles the size of standard wood chips may be fed to the prehydrolysis reactor if they can be successfully impregnated with acid. Thus, it should be possible to carry out the size reduction process on chips which have already had their fiber structure weakened by dilute acid hydrolysis. Such chips can be reduced in size with much smaller energy consumption. Engineering on the dilute acid pretreatment system is being carried out at Auburn University, where researchers are seeking methods of impregnating large chips with acid without the need to use large amounts of water (which would dilute the xylose produced in the prehydrolysis pretreatment).

A two stage process in which dilute acid removes the hemicellulose, and dilute sodium hydroxide next removes the lignin, is being also being investigated. Removal of the lignin simplifies the subsequent hydrolysis. Perhaps more importantly, the base treatment improves the rate of enzymatic hydrolysis by almost a factor of seven. Understanding this effect and how to take advantage of it could greatly improve hydrolysis processes. However, the base treatment also degrades part of the cellulose into organic acids, reducing the yield. The degree of degradation of the cellulose has been shown to be proportional to the severity of the dilute acid prehydrolysis which preceeds the alkali processing. Because of the primary importance of yield, cellulose

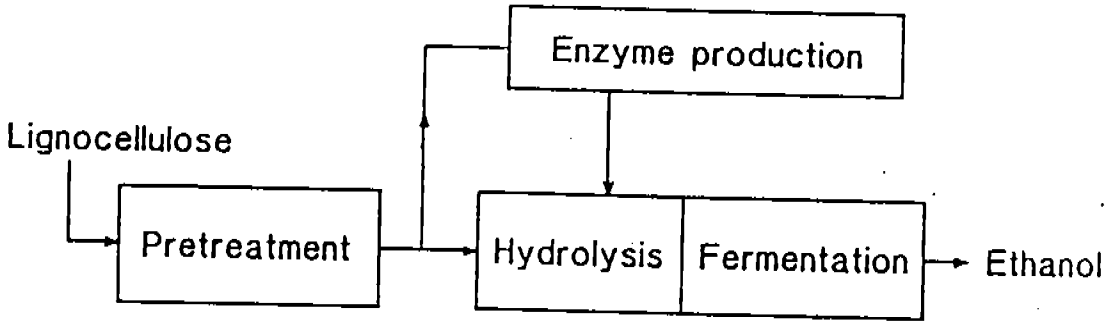

Figure 1. Process Schematic for Fungal Enzyme Hydrolysis

degradation must be overcome for this process to be useful.

The organosolv pretreatment simultaneously removes hemicellulose and lignin. In this process, the prehydolysis is carried out in the presence of both water and an organic solvent (either ethanol or methanol). Acid may be used to help catalyze the breakdown of the hemicellulose-lignin matrix, or the reaction may be carried out at higher temperatures without additional acid. In this case the reactions are catalyzed by the acetic acid liberated from the hemicellulose. The sugars from the hemicellulose are dissolved by the water, while the presence of the organic component allows the lignin to enter solution. After the lignin and sugars are washed from the reactor, the ethanol is evaporated, and the lignin is precipitated and removed from the water-sugar mixture by filtration. The lignin can be further processed to fuels, and the five and six carbon sugars produced in the hemicellulose hydrolysis can be fermented to ethanol, while the cellulose pulp which was left behind is readily digestible by enzymes. Organosolv is a true wood fractionation scheme, in which each of the three main components can be processed separately. The lignin is removed in a very reactive form which is amenable to further processing. Also, removal of the lignin greatly simplifies the subsequent enzymatic hydrolysis. However, these advantages come at the cost of increased capital and operating costs. In the coming year, a technical and economic evaluation will be made to determine the relative importance of fractionation and complexity.

While it has been shown that accessibility of the substrate to the enzyme is the most critical factor in enhancing enzymatic digestibility, there are several more subtle effects involving the structure of the lignocellulosic matrix. A greater understanding of these phenomena and their relationship to enzymatic hydrolysis are being studied under new subcontracts to Texas A&M and Colorado State University. These projects will selectively modify or remove constituents of the lignocellulosic matrix (acetyl groups, xylan, specific lignin bonds) to determine their individual effects on hydrolysis. Research at Dartmouth College also seeks improved understanding of this structure-digestibility, and will develop a model linking these effects to hydrolysis kinetics.

Integration of Feedstock Production and Conversion Processes

A new initiative is the integration of the feedstock production and the conversion process. While a processing facility using existing forest resources or agricultural residues will have to process whatever is available, the use of woody or herbaceous feedstocks grown specifically for use in a conversion facility opens new opportunities. These feedstocks can be tailored for improved performance and value in conversion. For example, the carbohydrate content of the biomass may be enhanced, or the digestibility by enzymes increased, or the ease of delignification improved by modifying the lignin composition. (While efforts to develop improved feedstocks for ethanol production is a new endeavor, such integrated systems of production and conversion have been studied for many years for anaerobic digestion processes.)

In the coming year, studies will be carried out on the effect of bark on enzymatic hydrolysis of bark. Bark constitutes a greater fraction of short rotation wood grown specifically for conversion, but is high in extractives, a natural defense against enzymatic attack. Further, screening studies will be carried out on short rotation forestry feedstocks and lignocellulosic herbaceous energy crops to identify both potential problems and valuable traits which could be improved by breeding and selection.

Simultaneous Saccharification and Fermentation (SSF)

The hydrolysis of cellulose is carried out by a complex of enzymes that have three

different modes of action (Figure 2) (Wood 1985). First, the endo-glucanase absorbs on the surface of the solid cellulose and attacks the interior of the polymer chain, splitting it and exposing two new chain ends. Next, exo-glucanases remove cellobiose units (two linked glucose units) from the non-reducing end of the cellulose chain. This process is further complicated because both the endo and exo enzymes occur in two different forms, probably because the beta-glycoside bond can occur in two different orientations (Eveleigh 1987). The cellobiose produced by the action of the exo enzyme can accumulate in solution and strongly inhibit the activity of the exo-glucanase. Finally, in a liquid-phase reaction, beta-G splits the cellobiose units into glucose. Similarly, the accumulation of glucose can inhibit the action of beta-G, causing a build-up of cellobiose, which again inhibits the exo-glucanase activity. Thus, the successful production of glucose (the desired feedstock for ethanol production) can cause severe end-product inhibition, which can greatly limit concentration, yield, or reaction rate.

The major focus of the Biochemical Conversion/Alcohol Fuels Program and SERI in-house research is the simultaneous saccharification and fermentation (SSF) process (Wyman, Spindler, and Grohmann 1987, Wright, Wyman and Grohmann 1987). To understand the rationale for SSF processes, it is useful to compare them to the separate hydrolysis and fermentation (SHF) (Wright and Power 1986). A cost breakdown for the SHF process is shown in Figure 3. The total cost of ethanol production is $2.66/gal, with the dominant contributions coming from feedstock (38% of the total) and enzyme production (25%). Feedstock costs total approximately $1.00/gallon, including both the $0.40/gallon for the cellulose which is converted to ethanol, and the $0.60/gallon for the feedstock which is burned to provide energy to run the process. A further $0.65/gal (25%) is contributed by enzyme production. An additional $0.40/gal (15%) is attributable to the hydrolysis reactor section. The dominant effect of yield comes from the fact that only 70% of the cellulose is converted to ethanol, and none of the other major fractions (xylose or lignin) are converted to fuels. The extremely high cost of enzyme production arises in part from the low rate of enzyme production caused by the use of an insoluble cellulose carbon source. However, a much more important cause is the high consumption of cellulase caused by the end-product inhibition of cellobiose and glucose. The higher the final glucose concentration, the higher the loading of a given cellulase is needed to achieve any given yield. Similarly, because the reaction is slowed or stopped by the presence of glucose and cellobiose, the hydrolysis is essentially halted before the reaction can proceed to completion. The optimal point for SHF is an enzyme loading of 20 IU/g substrate (33 IU/g cellulose), and a final glucose yield and concentration of 73% and 4.5%, respectively. Thus, end-product inhibition is in a large part responsible for the limitations in yield, product concentration, reaction rate, and high enzyme loading that give SHF such a high cost of production.

One means of alleviating this problem is to use cellulase preparations that have higher b-glucosidase activities. These newer enzyme preparations (such as Genencor 150L) are less inhibited by glucose and remove cellobiose more efficiently, allowing the reaction to proceed more swiftly to higher yields and glucose concentration (Wyman et al. 1986). Even further improvement can be made by continuously removing the glucose through fermentation (SSF). Using the yeast Candida brassicae, the enzyme loading can be reduced by almost a factor of five to 7 IU/g cellulose, the combined hydrolysis-fermentation increases to 79%.and the ethanol concentration is increased to 4.0% (equivalent to a glucose concentration of 8.9%, roughly twice that of SHF). This reduces the cost of ethanol production to $1.94/gal (Figure 4).

Realizing that cellobiose is an even greater inhibitor than is glucose, cellobiose-fermenting yeasts offer the potential for further improvement. Using Brettanomyces clausenii (a cellobiose-fermenting yeast) alone increased the yield to 83%, while a mixed

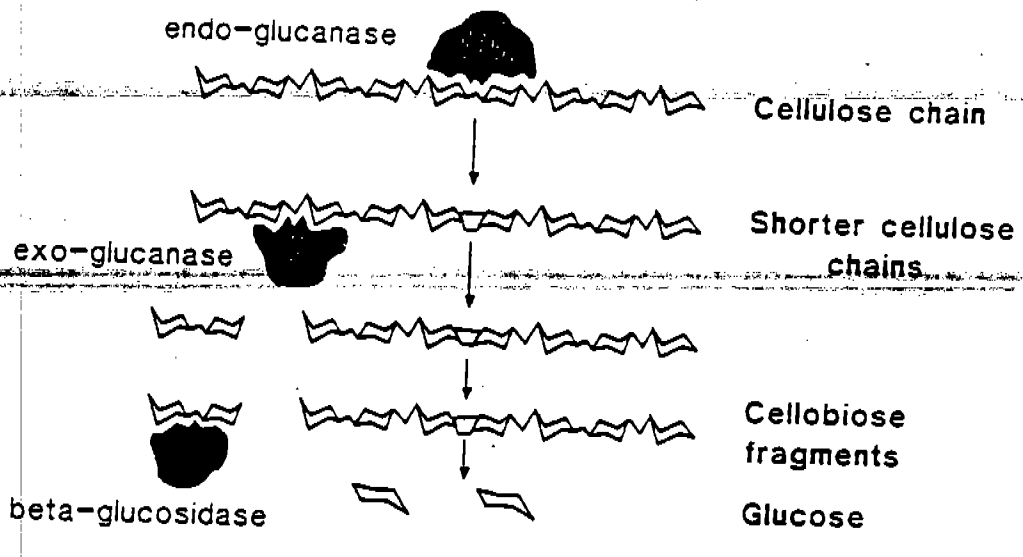

Figure 2. Mechanism of Enzymatic Hydrolysis and SSF

culture of Saccharomyces cerevisiae and B. clausenii increases the yield further. The B. clausenii is active early and removes the cellobiose inhibition when it is most important, providing high initial rates. The much more robust and ethanol-tolerant S. cerevisiae predominates in the latter stages of the reaction (Wyman et al. 1986). The mixed culture produces yields of 88% and ethanol concentrations of 4.5% from 10% cellulose, resulting in a predicted selling price of $1.78/gal (Wright et al. 1987). The achievement of these levels of performance in an SSF process is one of the most important achievements of the program in the past year.

Supporting the research on SSF performance at SERI is a project at the University of Colorado which is developing mathematical models of multiple substrate utilization by B. clausenii. An understanding of the utilization of multiple substrates is necessary because while cellobiose fermenting yeasts will ferment glucose and cellobiose, as well as other sugars which are present in biomass, they will utilize glucose to the exclusion of the other sugars if it is present in substantial amounts. Such models should point the way to develop operating strategies which rapidly and efficiently ferment all the sugars present. Another project investigating operating strategies is being carried out at Texas Tech, where alternative designs for continuous SSF are being evaluated.

The high power required to keep a viscous suspension of cellulose, lignin, and yeast has always plagued enzymatic hydrolysis. Recent research by Colorado State University on the effect of stirring on enzymatic hydrolysis of idealized substrates is finding that it may not be necessary to suspend the solids in order to achieve high reaction rates. It may be necessary only to prevent sugars from building up in some parts of the reactor while others are sugar free. If this is true, power requirements may be much less than previously assumed. Further, as sugars have little tendency to build up in an SSF reactor, only minimal mixing may be required in an SSF process.

Municipal solid waste (MSW) is a promising feedstock for conversion to ethanol because it is readily available at a negative cost. Pretreatments for MSW have not previously been studied at a scale which will provide meaningful operating data. CADCO, a joint project of United Biofuels, Foster-Wheeler, Raphael Katzen Associates, Riddick Engineering, and the University of Arkansas, is investigating the relationship between pretreatment and SSF using pilot or commercial scale equipment.

Cellulase Enzymology

The enzymes which catalyze the hydrolysis reaction are the heart of the SSF process, but many fundamental questions remain about their mechanism of action. Also, much of the improvement over the past decade is directly traceable to either the improvement of enzyme preparations, or improvements in our use of the enzyme which grew directly out of better understanding of their mechanisms of action. Thus, improvement of our understanding enzymology is an important component of the program.

Even with the low enzyme use of the SSF process, production of enzymes is expensive and requires use of carbohydrates which could be converted into ethanol. Thus, it would be useful to recycle the enzymes. Both the endo and exo components of cellulase have a high affinity for solid cellulose and may be recovered from the hydrolyzate solution by contacting the hydrolyzate with the fresh feed to the reactor. Alternatively, the enzymes which remain attached to the unreacted cellulose may be recycled by recycling the unreacted solids. However, because the beta-G component operates on the soluble sugar cellobiose, it has no affinity for cellulose, and is not recovered in the same process. In order to better understand these phenomena, a study was conducted to determine the affinity of the enzymes for various solids which may be present in the SSF

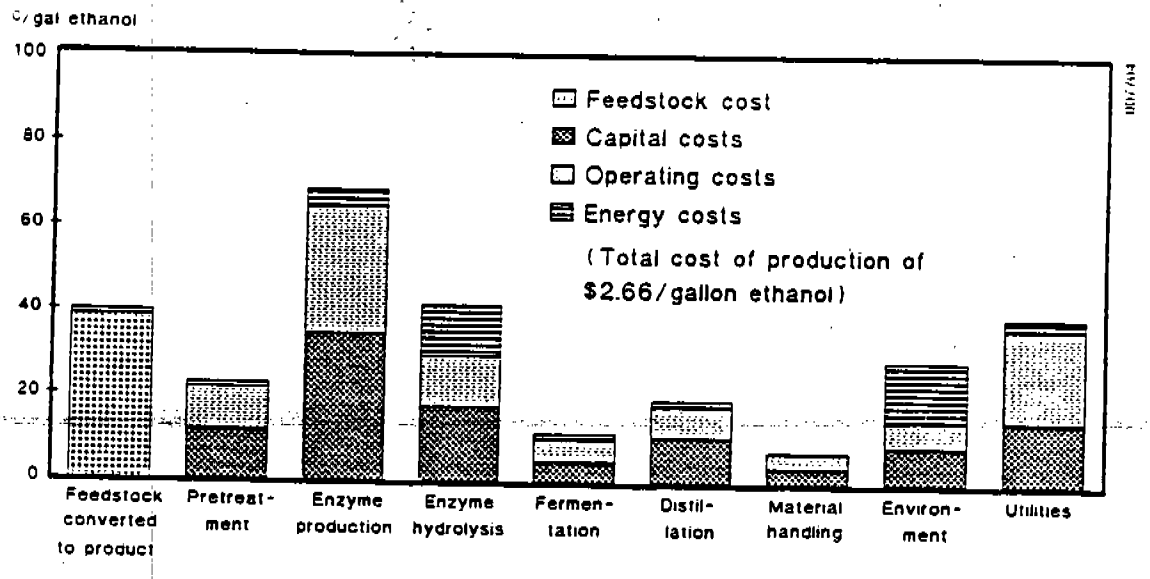

Figure 3. Breakdown of Ethanol Production Costs by Process Area for SHF Process

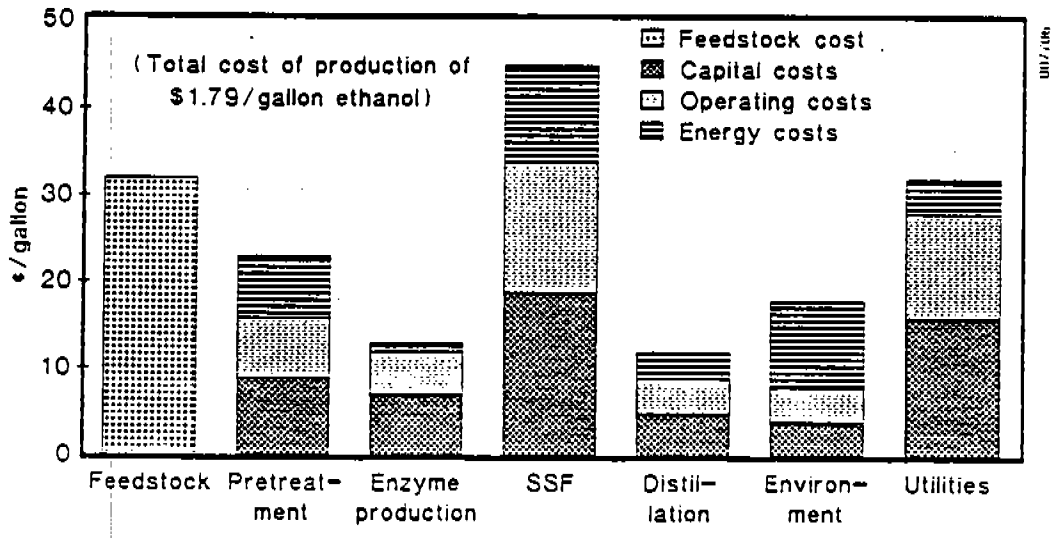

Figure 4. Breakdown of Ethanol Production Costs by Process Area for SSF

reactor output. While the endo and exo enzymes were found to have a high affinity for solid cellulosic materials (as expected), they had no large affinity for lignin. However, the beta-G component was strongly attracted to the lignin. Thus, lignin removal may be useful in order to maximize the activity and availability of the beta-G.

In order to recover the beta-G, research is being carried out to link the beta-G to the exo component so that they can be recycled simultaneously. Similar cross-linking technology is being investigated to stabilize the beta-glucosidase against thermal degradation. To date, beta-G components from several different sources have been purified and studied to better understand their native characteristics and thermal stability, and initial cross-linking experiments have been performed.

Cellulose recycle by absorbtion on ion exchange resins is being investigated in a joint project between Oak Ridge National Laboratory and SERI. Early investigations in this project have focused on measuring the activities of individual components of the enzyme mixture, and have yielded significant data on the interactions of the various components. A new project at Colorado State University will use monoclonal antibodies to attach colloidal gold to the exo and endo enzymes to determine if there is a physical association of these two components at the site of hydrolysis.

Researchers at Genencor will use genetic engineering techniques to better understand the structure of the active site of the exo cellulase enzyme cellobiohydrolase II. They will then use further genetic techniques to modify the structure of the enzyme to attempt to improve its activity. Changes will be made to test existing theories of active site structure. If the location of the active site is confirmed, changes will be made to the amino acid residues at the site in an attempt to improve activity. Also, the size of the enzyme will be greatly reduced to determine if a smaller enzyme can both retain its structure, and become more active because of its increased mobility. While earlier investigators have improved the activity of cellulase enzyme preparations, most of this improvement has occurred because of changes in the ratios of the various components. This is the first attempt to make specific and defined changes in the structure of a specific enzyme in mixture. Any improvements coming out of this research can be quickly commercialized because Genencor is the major U.S. producer of cellulase in a market where the primary competitors are European and Japanese.

Operation of hydrolysis processes at higher temperatures could improve reaction rates. SERI researchers have isolated a high temperature aerobic bacteria (Acidothermus cellulolyticus) which produces a cellulase system more stable than any discovered to date. This may prove valuable in its own right, or provide clues to the mechanisms of thermal stability which may be applicable to other cellulases. Improved production of enzymes with this organism are being developed by Colorado State University.

Enzyme Production

Cellulase enzymes are efficiently produced by the filamentous fungi T. reesei (Mandels 1981). Traditional methods of production use solid cellulose as both the inducer and the carbon source for enzyme production and growth. Although productivities of up to 150 IU/L-hr have been reported from such methods (McLean and Podruzny 1985), the productivities are limited by the low rate of hydrolysis (and hence low rate of growth) of the cellulose on the insoluble substrate. This low production rate makes enzyme production costs an important component of the overall processing cost. One promising alternative is to identify fungal mutants that produce enzymes while growing on soluble carbon sources such as lactose (Montenacourt 1983). However, lactose may not be available in the volumes required for producing enzymes for a major fuel industry. One

method of improving production rate is to decouple growth and enzyme production. SERI has investigated a procedure where the fungi are grown rapidly on the soluble sugar xylose, and then induced to produce cellulase by removing the xylose and providing only cellulase. A second approach is to improve the environment and cell density of the fungi by using fungi immobilized in a fluidized bed system (Colorado State University).

Another approach to improved enzyme production is to modify the organism. Researchers at Lehigh University are carrying out mutation selection procedures to create mutants which can grow and produce enzyme on soluble sugars such as glucose or xylose, as well as mutants with higher specific or beta-G activities. In the past year, mutant have been identified which produce significantly higher amounts of beta-G, and have the ability to produce enzymes while growing on glucose.

The classical theory of enzyme production postualtes that the machinery necessary for the production of an enzyme is always present in the cell, but is normally repressed. Induction occurs when a material in the environment is brought across the cell wall, binds to the repressor and inactivates it, allowing enzyme production to occur. Because a large, insoluble polymer such as cellulose cannot be brought across the cell wall, ther must be another molecule which inactivates the repressor. Researchers at Rutgers will try to identify the mechanism which regulates cellulase production within the fungi, so that cellulase production can be triggered independent of the external environment.

Conclusion

The Biochemical Conversion/Alcohol Fuels Program carries out research in pretreatment, hydrolysis and fermentation, and enzyme production. The success ofthis research has the potential to significantly reduce the cost of ethanol from lignocellulose.

References

ASCAF 1987 "La plate-forme experimentale de Soustons" ASCAF Association pour le Development des Carburants par la Fermentation, 92506 Rueil Malmasion, Cedex, France

Brownell, H. H., and J. N. Saddler, 1984, Steam-Explosion Pretreatment for Enzymatic Hydrolysis," Biotech. Bioeng. Symp. 14, p. 55.

Cooney, C.L., Demain, A.L., A.L. Sinskey, and D.I.C. Wang (July 1987) "Degradation of Lignocellulosic Biomass and Its Subsequent Utilization for the Production of Liquid Fuels." Subcontract Progress Reports for September 1981-August 1986 SERI/STR-231-3176 through 3180. Massachusetts Institute of Technology

Eveleigh, D.E. 1987 "Cellulase: A Perspective" Phil. Trans. R. Soc. Lond. A 321, 435-447

Fan, L. T., Lee, Y. H., and Gharpuray, M. M., 1982, Adv. Biochem. Eng. 23, pp. 158-187.

Grethlein, H. E., D. C. Allen, and A. O. Converse, 1984, "A Comparative Study of the Enzymatic Hydrolysis of Acid-Pretreated White Pine and Mixed Hardwood," Biotech. Bioeng. 26, p. 1498.

Grohmann, K., R. Torget, and M. Himmel, 1985, "Optimization of Dilute Acid Pretreatment of Biomass," Biotech. Bioeng. Symp. 15.

Holtzapple, M. T., and A. E. Humphrey, 1984, "The Effect of Organosolv Pretreatment on the Enzymatic Hydrolysis of Poplar," Biotech. Bioeng. 26, p. 670.

Lynd, L.R., R.H. Wolkin, and H.E. Grethlein 1986 "Continuous Fermentation of Avicel and Pretreated Mixed Hardwood by Clostridium thermocellum" in Biotechnology and Bioengineering Symposium No. 17. Eighth Symposium on Biotechnology for Fuels and Chemicals. ed C. Scott

Lynd, L.R. 1987 "Production of Ethanol from Lignocellulosic Materials Using Thermophilic Bacteria: Critical Evaluation of Potential and Review" (in press)

Mandels, M., 1981, "Cellulases," Ann. Reports Ferm. Proc. 5, pp. 35-78.

McLean, D., and M. F. Podruzny, 1985, "Further Support for Fed Batch Production of Cellulases," Biotechnol. Lett. 9, p. 683.

Montenacourt, B. S., 1983, Trichoderma reesei Cellulases," Trends Biotechnol. 1, pp. 156-161.

New Energy Development Organization 1987 "NEDO" Representative Office: Suite 924, 1800 K Street, N.W. Washington, D.C. 20006, USA

Spindler, D. D., C. E. Wyman, A. Mohagheghi, and K. Grohmann, 1987, Appl. Biochem. and Biotechnol. in press.

Wang, D.I.C., C.L. Cooney, A.L. Demain, R.F. Gomez, and A.J. Sinsky (July 1987) "Degradation of Lignocellulosic Biomass and its Subsequent Utilization for the Production of Chemical Feedstocks" Subcontract Progress Reports for March 1979 - February 1981. SERI/STR-231-3183 through 3185

Wood, T. M., 1985, "Properties of Cellulolytic Enzyme Systems," Biochem. Soc. Trans. 13, pp. 407-410.

Wright, J. D., C. E. Wyman, and K. Grohmann, 1987, "Simultaneous Saccharification and Fermentation of Lignocellulose: Process Evaluation," Appl. Biochem. and Biotechnol. in press.

Wright, J. D., P. W. Bergeron, and P. J. Werdene, 1987, "Progressing Batch Hydrolysis Reactor," I&EC Research 26, 699.

Wyman, C. E., D. D. Spindler, K. Grohmann, and S. M. Lastick, 1986, "Simultaneous Saccharification and Fermentation of Cellulose with the Yeast Brettanomyces clausenii," Biotech. Bioeng. Symp. 17, pp. 221-238.